

The University of Chicago

THE TOTAL AND
FREE CHOLESTEROL CONTENT OF
URINE FROM CANCER PATIENTS
AND NON-CANCEROUS
MEN AND WOMEN

A DISSERTATION SUBMITTED TO THE FACULTY
OF THE DIVISION OF THE BIOLOGICAL SCIENCES
IN CANDIDACY FOR THE DEGREE OF DOCTOR
OF PHILOSOPHY

DEPARTMENT OF BIOCHEMISTRY
DECEMBER, 1940

By
ROBERT STEELE

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Bokelmann and Muhlbock (1), in a recent review, find that there is no accord among various investigators as to whether blood cholesterol is raised, lowered or unchanged in cancer, and their own results indicate that in uterine cancer there is a slight lowering which can be detected only by averaging the results of a large number of cases. Even if a high degree of cholesterolemia accompanied cancer, there would still be no reason to expect increased cholesterol in the urine, since many independent observations have shown that cholesterolemia does not lead to cholesteroluria unless there is accompanying kidney damage (4, 5, 6, 7, 8, 9).

Consequently cholesteroluria in cancer would indicate some unusual mechanism operating in such cases. The present investigation was undertaken in an attempt to confirm the observations of Sobotka and Bloch (2, 3), which indicated that there might be a general increase in urinary cholesterol in cases of cancer.

Results and Methods of Previous Investigators

In nephrotic conditions cholesterol and cholesterol ester appear in the urine in such quantity that they are easily observed by chemical or even optical means. Thus doubly refractive cholesterol ester was observed at an early date in the sediments of nephrotic urines by means of the polarizing microscope (10), and chemical determinations of cholesterol have often proved successful in such nephrotic urines, which contain up to 100 mg. cholesterol in a 24 hour sample (5, 6, 7, 8, 11).

Normal urines, however, presented greater difficulties because of their extremely low content of cholesterol.

¹These investigations were supported in part by the National Advisory Cancer Council.

Gerard (12) in 1911 extracted 71 liters of normal urine by shaking it repeatedly in large flasks with ether, saponified the residue from the evaporated extracts and weighed the portion of the product which was soluble in dry ether. This crude product weighed 0.11 gm., gave the cholesterol color reactions, and yielded some crystals microscopically identified as cholesterol upon further purification.

Grunke (9) in 1922 evaporated urine to one-tenth its original volume, saponified the concentrate and adsorbed the cholesterol on calcium hydroxide precipitated in the saponification solution. The cholesterol was freed by dissolving the precipitate in hydrochloric acid, and extracted from the water suspension by shaking with chloroform. The Liebermann-Burchard reaction was run directly on the chloroform solution. By this method less than 1 mg. cholesterol in a 24 hour sample could not be quantitatively determined. The two normal urine samples investigated gave values of 1.1 mg., and less than 1 mg. respectively. A 92 per cent recovery of 4 mg. cholesterol added to a liter of normal urine was reported as an isolated test of the completeness of extraction.

Gardner and Gainsborough (11) in 1925 extracted filtered urine by adding 2 per cent of sodium hydroxide and shaking repeatedly during the course of four or five days with four or five one-half volume portions of ether. They saponified the residue from the combined ether extracts, precipitated the cholesterol from the non-saponifiable fraction with digitonin and weighed the dried precipitate. A second such ether extraction was run on the same urine sample after it had been evaporated to dryness, boiled under reflux with glacial acetic acid for 5 hours, diluted with water and made alkaline again. The claim was made that the boiling with acid liberated a form of cholesterol which was not extracted by the first shakings with ether. By this method the authors obtained 1.7 to 2.6 mg. per day total cholesterol in one pooled normal sample. In another they reported 2.8 to 4.2 mg. per day, of which 1.0 to 1.5 mg. were obtained only after glacial acetic acid hydrolysis.² No recovery experiments were reported.

²Actual figures reported are in milligrams per 100 cc. and have been calculated to milligrams per day by assuming an average daily urine volume of 1,000 to 1,500 cc.

Mirsky (13) precipitated the proteins of urine by means of tungstic acid, and extracted the adsorbed cholesterol from the dried precipitate with alcohol-ether mixture. He ran the Liebermann-Burchard reaction on the petroleum ether soluble fraction of the extract. As little as 200 to 400 cc. of urine could be used for the determination, and in the two normal urines studied values of 0.4 to 0.6 mg. per 24 hours and 3.0 to 4.5 mg. per 24 hours respectively were obtained.³ No recovery experiments were reported. Bruger (7), using Mirsky's method, obtained cholesterol values on six normal, filtered urines ranging from 0.0 to 1.1 mg. per 24 hours with an average of 0.5 mg. He also reported no recovery experiments. Sobotka, Bloch and Rosenbloom (3) applied the same procedure to a variety of pathological urines, and in particular to cancer urines. They found 61 cases of cancer in which urine cholesterol was below 0.1 to 0.15 mg. per day; 12 cases in which it ranged above this figure up to 0.5 to 0.75 mg.; and 19 cases in which it exceeded these figures and ranged up to a maximum of 5.5 mg. per day.⁴ Among miscellaneous pathological conditions (excluding cases of nephritis and nephrosis) 54 cases gave less than 0.1 to 0.5 mg.; 11 cases gave values between this and 0.5 to 0.75 mg.; and 9 cases gave upward of these values.⁵ No recovery experiments were recorded; agreement of 90 per cent between duplicates is claimed.

Butenandt and Dannenbaum (14) extracted 500 liters of acid-hydrolyzed urine with chloroform and separated cholesterol in pure form in a lengthy procedure. They reported the amount of cholesterol in normal male urine to be between 0.75 and 1.0 mg. per day by estimating the probable losses in the various steps of the isolation and adding these to the amount actually obtained.

Bloch and Sobotka (2) isolated about 0.4 mg. cholesterol per liter of pooled normal urine by extracting with n-butyl ether by vigorous stirring, saponifying the extract residue, and precipitating the non-saponifiable fraction with digitonin after separating as much cholesterol as possible by crystallization. By the same method they obtained about 4.0 mg. cholesterol per liter of pooled cancer urine. One hundred liter quantities of urine were used.

³See n. 2, p. 2.

⁴Ibid.

⁵Ibid.

From the above it is clear that no method of proven reliability for estimation of cholesterol and cholesterol ester in 24 hour urine samples has yet been devised.

Method Developed for the Extraction of
Cholesterol and Cholesterol Esters
from Urine

At first benzene extracts of urine were prepared in the Koch-Gallagher extractors, and the neutral fractions employed for androgen assays were used for the cholesterol estimations (15). However, due to the fact that the available extractors were being constantly and heavily used for other problems, an attempt was made to find some other extraction method which could be carried out with ordinary laboratory equipment. A method suggested by the Dingemans procedure (16) for sex hormone extraction was adopted temporarily. It involved refluxing benzene layered over the urine for a 12 hour period. Parallel extractions carried out with the Koch-Gallagher extractor and the refluxing method indicated that better yields of cholesterol were obtained in the latter procedure. However it was impossible to recover added cholesterol in anything like quantitative yields. This was found to be due to the fact that the commercial benzene used in the extractions contained a large amount of cholesterol-like material which masked the relatively small amount of cholesterol present in the urine. No attempt was made to identify this impurity in commercial benzene, but C.P. benzene which had been further purified by washing with concentrated sulfuric acid, alkali and water, and redistilling still gave a residue on evaporation to dryness which gave a precipitate with digitonin giving the Liebermann-Burchard reaction.

Consequently the use of benzene was abandoned, and ether was adopted as the extracting solvent. Ordinary U.S.P. ether gave very little residue on evaporation to dryness, and this residue contained nothing which gave a precipitate with digitonin. At first the ether was used in the same way as the benzene had been, but recovery experiments showed that this refluxing method gave very incomplete extraction when ether was used.

This led to the use of a mechanical shaking device in conjunction with ether as solvent. Erratic recovery results were obtained here too when 2 hour to 5 hour shaking periods were used.

Finally the rate of the extraction of normally present free and ester cholesterol as well as of added free and ester cholesterol was studied by making several successive short-time extractions with separate portions of ether. Twelve hours of continuous shaking were necessary for complete extraction, and added free and ester cholesterol, and normally present free and ester cholesterol were all extracted at the same rate. Subsequent experiments carried out with 12 hour shaking gave approximately 100 per cent recoveries of added cholesterol, so this length of time was adopted as the standard period for extraction.

When the benzene extracts prepared in the Koch-Gallagher extractor were used, it was necessary to fractionate the extracts in ether solution with alkali, and take the neutral fraction for analysis. This was necessary because of the large amounts of aqueous-alkali soluble material present in such extracts. In the benzene or ether extracts prepared by refluxing, this step was eliminated. Such extracts gave residues which were completely soluble in 1:1 acetone-absolute-alcohol.

Acid hydrolysis of the urine before extraction, a step which is necessary for the complete extraction of the sex hormones by fat solvents, did not increase the yield of cholesterol, and led to the saponification of any cholesterol acetate added to determine recovery of cholesterol ester.

The pH of the urine during extraction could be varied between 0.9 and 6.0 (no boiling in any case) without affecting the recovery of cholesterol or cholesterol ester, but at the lower pH values some urines gave extracts which were deeply colored and which gave colored digitonin precipitates, especially after potassium hydroxide saponification in the determination of total cholesterol. Such colored precipitates could not be determined colorimetrically.

As a result of these experiments the following was adopted as a standard procedure.

The 24 hour urine sample⁶ is measured and its pH taken.⁷ If the pH does not fall between 4.0 and 6.2 a small amount of

⁶Samples which had stood as long as three weeks at ice-box temperature have been used successfully.

⁷By quinhydrone method on a small portion of the sample.

acid or alkali is added to adjust it to a point in this range. If the total volume is 1,000 cc. or over, 1,000 cc. of the urine are placed in a two liter round bottom flask with a ground glass joint at the neck; if the volume is less than 1,000 cc. all of the sample is used plus enough water to make the total volume equal to 1,000 cc. The urine is then covered with 750 cc. of U.S.P. ether, the flask fitted with a ground glass stopper of a design described below and the flask shaken for 12 hours at a moderate speed on a machine. The stopper used is constricted at a point about 4 inches above the ground glass joint to a diameter of about $3/8$ inches. This constricted portion extends about 6 inches higher and has a small bulb blown in the middle of it. It is almost completely sealed at the upper end, but a very small hole is left to prevent pressure development inside the flask. This device is intended to prevent splashing of liquid ether out of the flask and to minimize evaporation without opposing the expansion of the ether which takes place whenever the room temperature rises.

After the 12 hour shaking the ether-urine mixture is allowed to stand overnight to separate, then is poured into a separatory funnel. The clear urine is run out of the funnel and discarded, the clear ether extract is decanted from the top of the funnel, and the emulsified layer which usually is present is retained in the funnel and shaken several times with about twice its volume of ether. The volume of this emulsion varies from sample to sample; when it is great more ether must be used, and more care taken to extract it completely than when it is small.

The combined extracts are evaporated to dryness on a steam bath in a flask with a ground glass joint at the neck, and using an all-glass condenser. The recovered ether is used again for extraction.

The residue from the evaporation is dissolved in about 100 cc. of 95 per cent alcohol and the alcohol removed under diminished pressure over a boiling water bath. This process is repeated once more to dry the residue further, then the contents of the flask are transferred through a taper-stemmed funnel to a 25 cc. volumetric flask, using 1:1 acetone-absolute alcohol as solvent. Four or five small portions of solvent are used in the transfer, and the flask and contents are warmed on the water bath during the process to facilitate solution.

Four cubic centimeters aliquots of this 25 cc. of acetone-absolute alcohol solution are taken for free and total cholesterol determinations in duplicate.

The Determination of Cholesterol
in the Urine Extracts

Except for minor modifications, the same procedure for determination of cholesterol was used as described by Schönheimer and Sperry (17).

C.P. acetone, glacial acetic acid, sulfuric acid, commercial absolute alcohol, and reagent grade acetic anhydride were used. The ether for washing the digitonin precipitates was U.S.P. ether purified after the directions of Schönheimer and Sperry (17); the digitonin solution was made up after the directions of these same authors, from Hoffman-LaRoche digitonin recrystallized by the method of Windaus (18).

Two cubic centimeters of the digitonin solution (containing 1 gm. in 500 cc.) are mixed with 4 cc. of 1:1 acetone-absolute alcohol containing the cholesterol in a 15 cc. centrifuge tube. If it is desired to determine total cholesterol, the 4 cc. of acetone-absolute alcohol solution of cholesterol are first mixed with 4 drops of a solution of potassium hydroxide (10 gm. plus 20 cc. water) and kept at 40° for 30 minutes, then neutralized with 5 per cent hydrochloric acid, using one drop of approximately .001 M brom-cresol purple as indicator. The solutions added to the tube are mixed with a fine-tipped stirring rod; care must be taken to ensure complete mixing of the potassium hydroxide solution, which tends to form a separate lower phase. Brom-cresol purple is used as indicator because the color of the urine extracts does not interfere with the observation of its color change. After the digitonin solution has been added, the tube, still containing the stirring rod, is kept in a covered Mason jar for about 16 hours, after which the precipitate is treated as follows.

First 1 cc. of a 1:2 acetone-ether mixture is layered over the contents of the tube,⁸ and the stirring rod is removed

⁸This modification of procedure was found necessary to avoid loss of precipitate in the case of urine extracts where the precipitated material sometimes collects at the surface of the solution, and is not centrifuged down completely by the ordinary method.

and placed on a rack. The tube is capped and centrifuged at a moderate speed for 10 minutes, then the supernatant liquid is decanted from the packed precipitate, 2 cc. of the 1:2 acetone-ether solution are flowed down the sides of the tube, the precipitate broken up with the same stirring rod originally present in the tube, and the contents of the tube recentrifuged. The washing of the precipitate is repeated twice again in the same way, but using pure ether in place of the acetone-ether mixture. After the final washing and centrifuging, the last wash solution is decanted, and the tube left in an inverted position on the rack for 30 to 60 minutes at 40° to dry. At the end of this time it is righted, the proper stirring rod inserted, and 3 cc. of glacial acetic acid are added. The tube is then placed in a sand bath at 60° to 80° and the precipitate broken up and agitated until it is dissolved. After cooling to room temperature, the resulting solution is ready for color development.

The tube is immersed in a beaker of water at 25° and 6 cc. of acetic anhydride and 0.3 cc. of concentrated sulfuric acid are added. The sulfuric acid is added from a fine-tipped 25 cc. burette which has been calibrated so that the number of drops in 0.3 cc. is known, and to avoid waiting for the viscous acid to drain, the requisite number of drops is counted out as recommended by Schönheimer and Sperry (17). The contents of the tube are then poured, after thorough mixing, into one of the colorimeter tubes with the aid of the stirring rod. The colorimeter tube is corked and placed in a water bath at 25° for 30 minutes in the dark, then removed, wiped dry, and read in the Evelyn colorimeter.

Tubes containing only the mixture of glacial acetic acid, acetic anhydride and sulfuric acid are always prepared along with those in which cholesterol is present. With one of these blank tubes inserted in the colorimeter, with the aperture set for the 6 cc. total volume, and with the No.620 Rubicon light filter inserted, the rheostat controlling the intensity of light falling on the photocell is adjusted so that the galvanometer swings to 100 on the scale. This represents 100 per cent transmission of the light, and the galvanometer reading obtained by removing the colorimeter tube (which possesses a light concentrating power due to its curved surfaces) is taken as the center reading to which the colorimeter is adjusted each time before a colored solution is introduced. When such a light absorbing solution is inserted,

the consequent galvanometer reading between 0 and 100 depends on the concentration of light absorbing material present. When the light is off, or a tube is inserted containing a solution which absorbs all the light, the galvanometer reading falls to 0.

The fraction of the light transmitted by the absorbing solution is equal to $G/100$, where G is the galvanometer reading, and since the concentration of absorbing material in the solution is inversely proportional to the logarithm of the fraction of the light transmitted, the following equations can be written, where C is the concentration of absorbing material and K_2 is the proportionality constant.⁹

$$C = K_2 \times \frac{1}{\log \frac{G}{100}} = K_2 \times \log \frac{100}{G} = K_2(2 - \log G)$$

The values for $2 - \log G$ for the various galvanometer readings are given in a table supplied with the instrument.

In standardizing the method and determining the value of the constant to be used in calculating cholesterol concentration from galvanometer reading, standard glacial acetic acid solutions of cholesterol containing 0.1 mg. cholesterol in 0.5 cc. were first used. These were measured out in quantities varying from 0.25 to 3.0 cc. and made to a total volume of 3 cc. with glacial acetic acid for color development. It was found that although separately prepared standard solutions gave results at a particular concentration agreeing to within 0.8 per cent when run simultaneously, and although the K_2 values for various concentrations of cholesterol always decreased by about 7 per cent from the 50 gamma to the 140 gamma cholesterol level; the agreement from day to day of readings obtained with the same standard solution, and at the same cholesterol concentration, was very poor, varying as much as 20 per cent over the whole period of the investigation. Some uncontrolled factor, such as variation in humidity of the air, may be responsible for some of the differences observed. The composition of the acetic anhydride was eliminated as an important factor in the changes by the fact that a sample of this

⁹ K_2 is used here to distinguish the constant from K_1 as described in the written material supplied with the colorimeter; $K_2 = 1/K_1$.

reagent, kept in a paraffin sealed container, gave results varying up to 10 per cent from day to day. The glacial acetic acid and concentrated sulfuric acid were kept in glass stoppered bottles, but no other precautions were taken to keep them dry.

In addition to the day-to-day variation in K_2 values observed with glacial acetic acid standards, a marked difference in the amount of color produced by the smaller quantities of cholesterol (50 to 150 gamma) was found when such quantities were first precipitated by digitonin, compared with the color produced by these same quantities dissolved directly in glacial acetic acid. Figure 1 shows the relationship between the two sets of K_2 values, the higher K_2 's in the case of the precipitated cholesterol representing lower color response to the same weight of cholesterol. A logical explanation for this difference is that the smaller quantities of cholesterol are incompletely precipitated by the digitonin.

Since it was desired to work within the range of from 50 to 150 gamma of cholesterol, it was necessary to use the K_2 values obtained with precipitated cholesterol standards. Furthermore, due to the day-to-day variations in color response to the same amount of cholesterol, mentioned above, it was necessary to carry out the following steps in comparing results obtained at various times with these standards.

One set of tubes containing 0.1 mg. quantities of cholesterol was included in every experiment. The K_2 values so determined at this concentration of cholesterol for all the experiments were assembled and averaged, and the average value taken as a reference point to which to correct the results of the experiments. When the various K_2 values obtained at different cholesterol concentrations in each particular experiment were changed by an amount proportionate to the deviation of the K_2 at 0.1 mg. for that experiment from the average K_2 at 0.1 mg., it was found that values for a given concentration of cholesterol obtained on different days came into agreement, so that the average deviations from the mean of individual ones of such values became very small. This indicates that the factor which causes the day-to-day variation of K_2 has a proportionate effect on the color produced at all cholesterol concentrations.

Figure 1 and Table 1 give the results obtained by treating the K_2 values calculated for many standardization experiments in

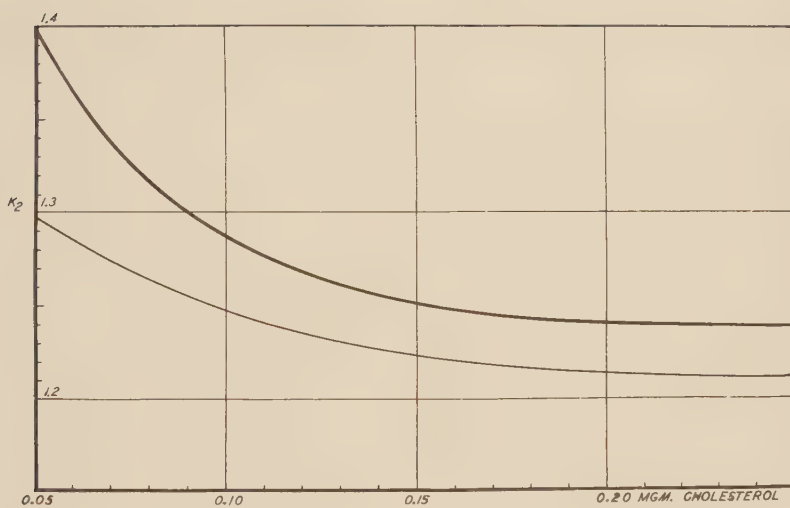


Fig. 1.--Average K_2 values obtained with acetone-absolute alcohol standards after digitonin precipitation (upper, heavy line) and with glacial acetic acid standards (lower, thin line), at various concentrations of cholesterol.

TABLE 1

AVERAGE DEVIATION OF INDIVIDUAL K_2 VALUES FROM THE MEAN K_2 VALUE
AT A GIVEN CHOLESTEROL CONCENTRATION AND THE PERCENTAGE
PRECIPITATION OF CHOLESTEROL BY DIGITONIN
(ALL AT VARIOUS CHOLESTEROL CONCENTRATIONS)

Mg. Cholesterol Present	Number of Individual Determinations	Average Per Cent Deviation of Individual K_2 Values from Mean K_2 Values	Average Per Cent Precipitation of Cholesterol by Digitonin*
0.050.....	19	± 3	93
0.075.....	25	± 3	96
0.100.....	23	± 1	97
0.125	24	± 2	98
0.135 to 0.495	18	± 2	98

*When the cholesterol is precipitated from 4 cc. of 1:1 acetone--absolute alcohol solution with 2 cc. of a watery solution of digitonin containing a total of 4 mg. digitonin.

the above manner. Figure 1 shows the corrected average values for K_2 at various levels of cholesterol concentration; column three of Table 1 lists the average deviation of individual values so corrected from the mean of the corrected values.

The percentage precipitation of cholesterol as the digitonide can also be obtained from the same series of experiments by using K_2 values observed with the glacial acetic acid standards and calculating the amounts of cholesterol actually contributing to the color; dividing these values by the amounts of cholesterol contained in the volumes of acetone-absolute alcohol standards¹⁰ measured out gives the percentage precipitation. The average percentage precipitation values at various cholesterol concentrations are given in column four of Table 1.

The procedure used in calculating the concentration of cholesterol in an unknown followed the same steps as that described above for comparing the K_2 values in the various experiments. A set of standards, each containing 0.1 mg. cholesterol in acetone-absolute alcohol was always prepared for precipitation

¹⁰These standards also contained 0.1 mg. cholesterol in 0.5 cc.

in parallel with unknowns. From this the current K_2 value at the 0.1 mg. level was obtained. The approximate concentration of cholesterol in the unknown solution was calculated from this value, and corrected by the following procedure. The average K_2 corresponding to this cholesterol concentration was read from the curve and multiplied by the ratio of the current K_2 at 0.1 mg. to the average K_2 at 0.1 mg. This final constant was used to recalculate the concentration of cholesterol in the unknown. This second approximation gave as accurate results as required, since nearly all of the unknowns determined were found to contain between 75 and 125 gamma of cholesterol. In this range the roughly calculated cholesterol concentrations vary from the more exactly calculated concentrations by only a few per cent. The mathematical expression for the final constant used for an unknown is as follows:

$$K_2(F) = \frac{K_2(C)}{K_2(A)} \times K_2(X)$$

where $K_2(C)$ is the current constant at the 0.1 mg. level, $K_2(A)$ is the average of constants at the 0.1 mg. level as read from the standard curve, and $K_2(X)$ is the average of constants at the approximate cholesterol level of the unknown.

Discussion of the Results of the Determinations of Cholesterol in Urine

Table 2 illustrates the dependability of the extraction method as shown by the agreement between duplicates and by the percentage recoveries of added cholesterol and cholesterol acetate. Tables 3, 4, and 5 give the values for free and total cholesterol obtained for non-cancer male urines, non-cancer female urines and cancer male urines respectively.

Since hospital urine collections had to be relied upon in all the cancer male cases, and in a large number of the non-cancer female cases, an attempt was made to estimate the completeness of such collections by determining 24 hour creatinine values for the samples in question. It was found, however, that the creatinine values so obtained were very low in nearly all the cancer cases, and also in the non-cancer male and female cases in which the donor had been hospitalized for some disorder other than cancer. This was found to be true even in samples obtained from a hospital

TABLE 2

DEPENDABILITY OF URINE EXTRACTION METHOD AS DEMONSTRATED BY AGREEMENT
BETWEEN DUPLICATES AND BY RECOVERIES OF ADDED FREE AND ESTER
CHOLESTEROL

Number	Initials* of Donor	Mg. Free Cholesterol Added	Mg. Cholesterol Added as Acetate	Mg. Free Cholesterol Found	Per Cent Deviation between Duplicates	Mg. Free Plus Ester Cholesterol Found	Per Cent Deviation between Duplicates	Per Cent Recovery of Added Free Cholesterol	Per Cent Recovery of Added Free Plus Ester Cholesterol
1a	Mr. J.C.	0.0	0.0	1.21	±2	Not det'd.	..	..	..
1b	Mr. J.C.	0.0	0.0	1.23				..	..
2a	Mr. V.C.	0.0	0.0	0.92	±5	Not det'd.	..	..	..
2b	Mr. V.C.	0.0	0.0	0.87				..	..
9a	Mr. Val.	0.0	0.0	0.58	±3	0.69	±4	..	..
9b	Mr. Val.	0.0	0.0	0.60		0.66		..	..
10a	Mrs. McC.	0.0	0.0	1.18	±4	1.29	..	..	..
10b	Mrs. McC.	0.0	0.0	1.22		1.29		..	..

IIIa	PM-RS	0.0	0.0	0.59	..	Not det'd.	..	98	...
IIIb	(pooled)	0.510	0.0	1.09	..				
3a	Mr. R.V.	0.0	0.0	0.71	..	0.77	..	100	103
3b	Mr. R.V.	0.255	0.218	0.97	..	1.28	..		
4a	Miss M.K.	0.0	0.0	0.55	..	0.74	..	85	114
4b	Miss M.K.	0.255	0.218	0.77	..	1.28	..		
7a	Mrs. M.	0.0	0.0	0.60	..	0.88	..	107	96
7b	Mrs. M.	0.255	0.218	0.88	..	1.53	..		
11a	Miss P.	0.0	0.0	0.39	..	0.39	..	88	94
11b	Miss P.	0.255	0.218	0.62	..	0.83	..		
14a	Mr. J.R.C.	0.0	0.0	0.28	..	0.28	..	85	70
14b	Mr. J.R.C.	0.225	0.218	0.48	..	0.59	..		
44a	Mr. R.V.	0.0	0.0	0.61	..	0.58	..	120	95
44b	Mr. R.V.	0.225	0.218	0.89	..	1.00	..		
45a	Mr. R.S.	0.0	0.0	0.73	..	0.71	..	104	86
45b	Mr. R.S.	0.225	0.218	0.97	..	1.09	..		
46a	Mr. P.M.	0.0	0.0	0.78	..	1.03	..	108	93
46b	Mr. P.M.	0.225	0.218	1.03	..	1.44	..		

*Equal quantities of the same urine sample, usually a 48-hour sample, are taken for a and b in each case, each portion diluted to 1,000 cc., with water, and the pH adjusted to 4.5 to 6.0 before extracting by shaking 12 hours with 750 cc. ether.

TABLE 3
FREE AND TOTAL CHOLESTEROL VALUES IN 24-HOUR URINE SAMPLES
OF NON-CANCER MALES

Sample No.	Initials of Donor	Mg. Total Cholesterol	Mg. Free Cholesterol	Mg. Ester Cholesterol by Difference	Pathology (If Any)
1.....	J. C.		1.20		Normal
2.....	V. C. 1		0.89		Normal
3.....	R. V. 1	0.97	0.90	0.07	Normal
9.....	Mr. V.	0.69	0.58	0.11	Hyperthyroid
19.....	Mr. Sch.	0.87	0.84	0.03	Diabetes M.
41.....	J. R. C.	0.71	0.71	0.0	Normal
42.....	E. H. 2	0.48	0.55	-0.07	Normal
44.....	R. V. 2	0.58	0.59	-0.01	Normal
45.....	R. S.	0.82	0.84	-0.02	Normal
46.....	P. M.	1.03	0.78	0.25	Normal
55.....	Mr. Sh.	0.75	0.73	0.02	Normal
56.....	Mr. W.	0.76	0.75	0.01	Normal
57.....	G. B.	0.73	0.69	0.04	Normal
58.....	Mr. We.	0.70	0.70	0.0	Normal
59.....	Mr. K.	0.91	0.88	0.03	Normal
62.....	Mr. C.	0.68	0.72	-0.04	Normal
63.....	Mr. N.	0.63	0.57	0.06	Normal
64.....	Mr. Sm.	0.66	0.65	0.01	Normal
65.....	Mr. B.	0.70	0.70	0.0	Normal
66.....	Mr. Su.	0.70	0.65	0.05	Normal
Average..		0.74	0.71	0.03	

TABLE 4
FREE AND TOTAL CHOLESTEROL VALUES IN 24-HOUR URINE SAMPLES
OF NON-CANCER FEMALES

Sample No.	Initials of Donor	Mg. Total Cholesterol	Mg. Free Cholesterol	Mg. Ester Cholesterol by Difference	Pathology (If Any)
4.....	Miss K. ¹	1.49	1.11	0.38	Normal
7.....	Mrs. Ma.	1.76	1.20	0.56	Hypertension
10.....	Mrs. McC.	1.29	1.18	0.11	Paratyphoid
11.....	Miss Pr.	1.15	1.12	0.03	Pneumonia
14.....	Mrs. May	1.07	1.00	0.07	(B. coli) Pyelitis
15.....	Mrs. Ko.	0.73	0.64	0.09	Normal
16.....	Miss Z.	1.33	1.48	-0.15	Peptic ulcer
48.....	Miss So.	1.29	1.18	0.11	Hyperthyroid
50.....	Mrs. Mi.	2.38	2.22	0.16	?
51.....	Miss K.K.	1.56	1.43	0.13	Normal
52.....	Miss K. ²	1.00	0.87	0.13	Normal
53.....	Mrs. Po.	0.54	0.52	0.02	?
54.....	Miss T.	1.09	0.72	0.37	?
60.....	Miss X.	1.30	1.24	0.06	Normal
61.....	Miss Ow.	0.62	0.62	0.0	Scarlet fever
Average..		1.18	1.04	0.14	

TABLE 5
FREE AND TOTAL CHOLESTEROL VALUES IN 24-HOUR URINE SAMPLES
OF MALES WITH CANCER

Sample No.	Initials of Donor	Mg. Total Cholesterol	Mg. Free Cholesterol	Mg. Ester Cholesterol by Difference	Pathology (Location of Cancer)
17.....	Mr. P.	1.15	1.00	0.15	Rectum
20.....	Mr. Y.	0.52	0.51	0.01	Larynx
21.....	H-13	0.83	0.71	0.12	Rectum
22.....	H-8	0.64	0.64	0.0	Tongue
23.....	H-16	0.78	0.74	0.04	Lymph nodes
24.....	H-9	0.93	0.96	-0.03	Lung
25.....	H-20	0.59	0.57	0.02	Stomach
26.....	H-15	0.58	0.50	0.08	Lung
27.....	H-14	0.89	0.83	0.06	Rectum
28.....	H-18	0.85	0.77	0.08	Lymph nodes
29.....	H-2	0.64	0.64	0.0	Lip
30.....	H-17	0.82	0.80	0.02	Stomach
33.....	H-21	0.76	0.70	0.06	Stomach
34.....	H-1	1.23	1.12	0.11	Lip
35.....	H-3	0.70	0.70	0.0	Lip
36.....	H-4	0.79	0.79	0.0	Lip
37.....	H-7	0.63	0.59	0.04	Stomach
38.....	H-10	0.67	0.60	0.07	Esophagus
39.....	H-19	0.70	0.68	0.02	Stomach
40.....	H-5	1.08	1.05	0.03	Esophagus
Average..		0.80	0.75	0.05	

unit where complete urine collections were known to be stringently insisted upon. A similar effect in cases of prolonged illness is described by Peters and Van Slyke (19). It was decided that the average urine volumes for the various types of cases studied was a more reliable index of the completeness of urine collection.

Nearly all the non-cancer male samples were from normal males connected with the department and whose collections could be depended upon for completeness. Since the average urine volume in this group is no greater than the average volume in the cancer male group, and is less than the average for the non-cancer female group, it seems probable that collections were complete in these other groups also.

In Table 6 are given the ranges and average values compiled from Tables 3, 4, and 5 for the different groups, and also the average urine volumes. It is observed that non-cancer males and cancer males do not differ in their cholesterol excretion, and that non-cancer females excrete nearly twice as much cholesterol and cholesterol ester as do males. There is practically no ester cholesterol in non-cancer male or cancer male urine, but a significant amount is present in non-cancer female urine.

With regard to this point it should be stated that difficulty is sometimes encountered in determining free plus ester cholesterol values, in that the digitonin precipitates obtained from saponified extracts sometimes form a sticky mass during the first two washings. This would tend to make washing less efficient, and wider variations in color response would be expected due to the possibility of occlusion of either foreign chromogenic substances or of color inhibiting substances such as water. The values for total cholesterol, then, are probably not as dependable as those for free cholesterol, and indeed it is sometimes found that they fall below the figures for free cholesterol in the same extract. However the recovery studies on ester cholesterol show that 0.2 mg. of this material can be determined with an average accuracy of 11 per cent when present along with 0.5 to 0.9 mg. free cholesterol, and since the average non-cancer female urine contains 0.14 mg. of ester cholesterol and about 1.04 mg. of free cholesterol, it seems likely that the average amount of ester cholesterol in female urine has been fairly accurately determined.

The question remains as to whether the values for cholesterol above represent true cholesterol, or are in whole or in

TABLE 6

AVERAGE VALUES FOR 24-HOUR FREE AND TOTAL CHOLESTEROL AND 24-HOUR URINE VOLUME
FOR NON-CANCER MALES, NON-CANCER FEMALES, AND MALES WITH CANCER

Type of sample	Mg. Total Cholesterol; Av./24 Hrs.	Mg. Free Cholesterol; Av./24 Hrs.	Mg. Ester Cholesterol; Av./24 Hrs.	Cc. Urine Av./24 Hrs.	Number of Cases
Non-cancer males	0.74 Range 0.48-1.03	0.71 Range 0.55-1.20	0.03 Range 0-0.25	1,200 Range 660-3,000	18
Non-cancer females	1.18 Range 0.54-2.38	1.04 Range 0.52-2.22	0.14 Range 0-0.37	1,500 Range 700-2,540	15
Cancer males	0.80 Range 0.52-1.23	0.75 Range 0.50-1.12	0.05 Range 0-0.15	1,200 Range 620-3,000	20

part due to other compounds of similar chemical structure. The twofold specificity of the method used, involving both digitonin precipitation and the Liebermann-Burchard reaction makes it unlikely that any compound present in urine other than one of a steroid nature would interfere. Among these compounds only those with a hydroxyl group at C_3 having the same steric configuration as cholesterol would be precipitated as far as is known (20), and of these only the unsaturated compounds would give the color reaction.

Androsterone, isoandrosterone, isodehydroandrosterone, testosterone, dihydrocholesterol, epi-allo-pregnanolone, pregnandiol, a-androstenediol-17 cis and b-androstenediol-17 trans were all tested in concentrations of 0.1 mg. in the 4 cc. of acetone-absolute alcohol, both alone and in the presence of 0.1 mg. cholesterol in every case. Of all these, only dihydrocholesterol gave a precipitate with digitonin in the absence of cholesterol, and none affected the color response given by the 0.1 mg. of cholesterol precipitated from the same solution. Three of the above compounds besides dihydrocholesterol have the correct configuration at C_3 for precipitation, namely isoandrosterone, isodehydroandrosterone, and b-androstenediol-17 trans, but are apparently not precipitated under the conditions employed by us for precipitation of cholesterol. The experiment was repeated in the cases of androsterone, isodehydroandrosterone, and isoandrosterone, but using 2.0 mg. of each, both alone and in conjunction with 0.1 mg. quantities of cholesterol. The isoandrosterone was precipitated under these conditions, but the precipitate gave no color with the Liebermann-Burchard reagents, and the color value for the 0.1 mg. of cholesterol precipitated simultaneously was not altered by the presence of the digitonide of isoandrosterone. The androsterone and isodehydroandrosterone were not precipitated even in this relatively high concentration, nor did they interfere with the precipitation and color development of cholesterol present in the same solution.

In considering the possibility of interference by these compounds, several things must be taken into account. For instance it is known that androgenic activity is present in the urine largely in the combined form; the combination must be broken by acid hydrolysis before the androgens are extractable by fat solvents. This alone makes it unlikely that they would interfere

with the cholesterol determinations. If it be assumed that these compounds were completely extracted however, the following considerations show that they still would not be present in sufficient concentration to interfere.

The range of androgen excretion in normal urine is given as 30 to 100 international units per day (21). Callow and Callow (22) find that isodehydroandrosterone, the only one of the known androgens which would give the Liebermann-Burchard reaction is present in normal urine only to the extent of 0.2 mg. per liter. According to this figure, the amount of this compound which would be present in one of our aliquots would be about 0.04 mg. However the total amount of androgenic activity accounted for by all the compounds isolated by Callow and Callow is only about 28 international units per day, or less than half of the average total number given above, so assuming that the isolation of isodehydroandrosterone by Callow and Callow was incomplete, and arbitrarily setting the amount of isodehydroandrosterone at a figure such that half of the total androgenic activity would be accounted for by it alone, we find that the amount which would then be present in one of our 4 cc. aliquots would be 1.7 mg. Since 2.0 mg. of the pure substance failed to interfere, it is safe to say that none of the known androgens if present in normal concentration would affect the cholesterol determination. However some as yet undiscovered androgen, or some non-androgenic steroid other than cholesterol might be contributing to the values observed. The estrogens can be disregarded because of their low concentration in normal urine.

The presence of a small quantity of blood in a urine sample would greatly alter the amount of cholesterol determined, since the concentration of cholesterol in blood is so high in comparison with its concentration in urine. For example 0.1 cc. of blood in a 1 liter urine sample would raise the total cholesterol of the sample by 0.2 to 0.3 mg. It was found by experiment that the smallest amount of blood detectable by the benzidine reaction as carried out by us (23) was 0.05 cc. per liter of urine, so that not more than 0.1 to 0.15 mg. total cholesterol can be due to blood in a urine sample if the sample gives a negative benzidine reaction. This reaction was run routinely on all urine samples except for a few at the first of the series, and all samples giving positive reactions were discarded.

Unfiltered urines were used in all the experiments because it did not seem obvious that sedimentary cholesterol would represent only the amount due to the cell debris present and not also some which had been adsorbed by the sediment from the supernatant solution. The amount of cholesterol in urinary sediment has been studied in cases of nephrosis and in one normal case by Bruger (7), who found 47 per cent of the total cholesterol of the normal urine in the sediment. The absence of ester cholesterol in our male samples indicates that no significant amount of cellular material was present in them. On the other hand, cellular elements might be responsible for both the ester cholesterol and the increased total cholesterol of the female urines.

Summary

1. A method for free and ester cholesterol in 24 hour urine samples has been devised, and its accuracy determined by experiments involving recovery of known amounts of cholesterol and cholesterol acetate added to the samples before extraction.
2. The average daily urinary excretion of free and ester cholesterol has been determined for non-cancer females, non-cancer males, and cancer males.
3. No difference has been found between non-cancer males and cancer males in urinary cholesterol excretion. About 0.7 mg. free cholesterol and no ester cholesterol are excreted per day by each group on the average. Non-cancer females excrete about 1.0 mg. of free cholesterol and about 0.1 mg. ester cholesterol per day.
4. The specificity of the method for cholesterol, and the significance of the results have been discussed.

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